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PATENTS, TRADEMARKS, COPYRIGHTS AND RELATED MATTERS

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July 6, 2000

UTILITY PATENT APPLICATION TRANSMITTAL
(new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket Number: SGM 6934.1
First Named Inventor: Brian W. Ward et al.
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APPLICATION ELEMENTS

1. [X] Fee Transmittal Form (original and duplicate)

2. [X] Specification [Total Pages 38]

3. [X] Drawings [Total Sheets 18]

4. Oath or Declaration [Total Pages 4]

   a. [ ] Newly executed (original or copy)
   [X] New (unexecuted)

   b. [ ] Copy from a prior application (for continuation/divisional with Box 17 completed)
   i. [ ] DELETION OF INVENTOR(s) Signed statement attached deleting inventor(s) named in prior application.

5. [ ] Incorporation By Reference (useable if Box 4b is marked)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. [ ] Microfiche Computer Program (Appendix)

7. [ ] Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
   a. [ ] Computer Readable Copy
   b. [ ] Paper Copy (identical to computer copy)
   c. [ ] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. [ ] Assignment Papers (cover sheet & document(s))

9. [ ] 37 CFR 3.73(b) Statement [ ] Power of Attorney

10. [ ] English Translation Document (if applicable)

11. [ ] IDS with PTO-1449 [ ] Copies of IDS Citations

12. [ ] Preliminary Amendment

13. [X] Return Receipt Postcard

14. [ ] Small Entity Statement(s)
    [ ] Statement filed in prior application; status still proper and desired

15. [ ] Certified Copy of Priority Document(s) if foreign priority is claimed

16. [ ] Other: ______________________________

IF A CONTINUING APPLICATION, CHECK APPROPRIATE BOXES AND SUPPLY THE REQUISITE INFORMATION

17. [ ] Continuation [ ] Divisional [ ] Continuation-in-Part of prior application No.: __________

    [X] Complete Application based on provisional Application Nos. 60/143,009, filed July 9, 1999.

CORRESPONDENCE ADDRESS

18. Correspondence Address: Customer Number 321
    Attention: Edward J. Hejlek

Respectfully submitted,

Edward J. Hejlek, Reg. No. 31,525

EJH/dep

FEE TRANSMITTAL

Application Number not yet assigned
Filing Date July 6, 2000
First Named Inventor Brian W. Ward et al.
Attorney Docket Number SGM 6934.1

METHOD OF PAYMENT

1. [ ] The Commissioner is hereby authorized to charge the indicated fees to Deposit Account No. 19-1345, in the name of Senniger, Powers, Leavitt & Roedel.

   [ ] The Commissioner is hereby authorized to charge any additional filing and claim fees under 37 CFR 1.16 and application processing fees under 37 CFR 1.17 to Deposit Account No. 19-1345, in the name of Senniger, Powers, Leavitt & Roedel.

2. [X] Check Enclosed. The Commissioner is hereby authorized to charge any under payment or credit any over payment to Deposit Account No. 19-1345, in the name of Senniger, Powers, Leavitt & Roedel.

FEE CALCULATION

1. [X] BASIC FILING FEE $ 760.00 (Type: Formal Utility)
   Entity Status: large

2. [X] CLAIM FEE $312.00

   Total Claims 33
   Independent Claims 4
   Multiple Dependent Claims ______

3. [ ] ADDITIONAL FEES $________

   [ ] Surcharge - late filing fee or oath
   [ ] Surcharge - late provisional filing fee or cover sheet
   [ ] Extension for reply within ________ month
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TOTAL AMOUNT OF PAYMENT $ 1,072.00

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Edward J. Hejlek, Reg. No. 31,525 Date

EJH/dep

TRACER REAGENTS THAT ENHANCE REACTION-PRODUCT ANALYSIS

REFERENCE TO RELATED APPLICATIONS

This application is a non-provisional application claiming priority from provisional application serial no. 60/143,009 filed July 9, 1999.

BACKGROUND OF THE INVENTION

The present invention generally relates to reagents that are essential for an enzymatic reaction and that enhance reaction-product analysis. In specific, preferred embodiments, the invention provides compositions of essential components which facilitate subsequent chromatographic or electrophoretic analysis.

Enzymes are frequently used in laboratories to catalyze a variety of transformations. Typical enzymes which have been utilized include proteases, peroxidases, oxidases, kinases, amylases, and several nucleic acid modifying enzymes such as DNA polymerases, RNA polymerases, ligases, kinases, restriction endonucleases, phosphodiesterases, DNases, exonucleases, RNases, and phosphatases. The nucleic acid-modifying enzymes have been frequently used in molecular biology laboratories as part of procedures such as polymerase chain reaction ("PCR"), sequencing, southern hybridization analysis, restriction endonuclease analysis, RNase protection, and the production of labeled probes.

The steps involved in performing an enzyme catalyzed transformation can generally be categorized as reaction mixture formulation, enzymatic reaction, reaction product characterization, and reaction product use. The steps of mixture formulation, product characterization, and product use are labor intensive. The formulation of enzyme reaction mixtures entails combining reaction components which are essential for the enzymatic reaction

into a reaction mixture. The reaction mixture is then incubated under conditions favorable for the enzymatic reaction to take place, and for a time sufficient to allow the enzymatic reaction to proceed substantially to completion. The reaction mixture is typically analyzed to evaluate the characteristics of the products formed. This analysis often entails a chromatographic or electrophoretic procedure to separate and evaluate the reaction products, and to determine whether the enzymatic reaction has proceeded to completion. Downstream applications entail a wide variety of varied uses for the products of enzymatic reactions, such as utilization in manufacturing, and further processing of the product with enzymes or chemical processes. In the case of molecular biological enzymatic reactions, examples of downstream applications are transformation of prokaryotic or eukaryotic cells, detection of complementary sequences by southern or northern hybridization, sequencing, phosphorylation, dephosphorylation, ligation, restriction digestion, endonucleolytic digestion, exonucleolytic digestion, and purification.

Procedures such as liquid chromatography and polyacrylamide gel electrophoresis ("PAGE") have been frequently used to analyze the results of the enzymatic reactions by separating the reaction products by, for example, molecular weight. The results of the modification of nucleic acids by enzymes such as DNA polymerase have typically been analyzed by subjecting the reaction products to electrophoresis through polyacrylamide or agarose gels.

To analyze enzymatic reaction products using chromatography or electrophoresis, the sample to be analyzed has often been combined with components which assist the operator in performing the separation. One such component is a "tracer", which is a detectable

moiety such as a dye which is generally added to the sample immediately before loading the sample onto the chromatography column or electrophoresis gel. The tracer migrates in the medium in the same direction as the sample to indicate the progress of the separation.

Another reagent, termed "high density agent" herein, has also been commonly utilized in electrophoretic analysis of the enzymatic reaction products. High density agents are generally water soluble, dense liquids, such as a solution of sucrose or glycerol, which have been mixed with the sample, usually after the enzymatic reaction is complete, to increase the sample density. The increased density of the sample resulting from mixing the sample with the high density agent aids, for example, in loading the sample into a well of an electrophoresis gel by allowing the sample, when pipetted into the top of the well, to "fall" through the less-dense electrophoresis buffer solution to the bottom of the well.

High density agent and tracer have been combined with reaction product-containing samples to be electrophoretically separated. The combination of high density agent and tracer is generally termed "loading buffer".

While tracers and loading buffers have usually been mixed with the sample <u>after</u> the enzymatic reaction is complete, as diagramed in Fig. 1, their use <u>before</u> the commencement of nucleic acid-modifying enzymatic reactions is also known, as diagramed in Fig. 2. Hoppe et al., <u>BioTechniques</u> 12:679-680 (1992) describe combining a solution of sucrose (up to 30%) and certain dyes (cresol red, tartrazine, or yellow food coloring #5) with an enzyme reaction mixture containing all other components for PCR. After the PCR procedure, the samples were reportedly loaded directly onto an agarose gel for

electrophoretic analysis. The authors noted that several dyes and heavy components were inhibitory to the Taq polymerase enzyme used, but that sucrose, cresol red, tartrazine, and yellow food coloring #5 were compatible with Taq.

Certain commercially available products provide tracer or loading buffer for use in enzyme reaction mixes for PCR. A thermostable polymerase preparation, Red Hot DNA Polymerase, is available from Advanced Biotechnologies and reportedly contains a red dye for use to indicate enzyme addition in the enzyme reaction mixture. There are also two products available which comprise a red tracer and a high density agent, for addition to a PCR reaction mixture before amplification. One, called *Redi*Load, is available from Research Genetics, Inc., and the other, called Rapid-Load™, is available from OriGene Technologies. These reagents must be added to the reaction mixture in a separate pipetting step.

SUMMARY OF THE INVENTION

Despite the efforts and improvements made in the prior art, inefficiencies in reaction mixture formulation and reaction product analysis still exist. In particular, there is no product to date which combines an essential component for an enzyme reaction with a tracer and/or high density agent which can be used in an enzymatic reaction and provide sufficient tracer and/or high density agent such that the product of the enzyme reaction could be directly evaluated in a chromatographic or electrophoretic procedure without supplying additional tracer or high density agent. Such a product ("analysis reagent composition") would provide additional advantages over the products currently available because it would (1) indicate reagent addition into the enzymatic reaction

mix, and (2) eliminate the need for separately adding a loading buffer since the loading buffer components are added along with the essential reagent.

Among the several objects of the invention, therefore, is the provision of compositions for use in formulating enzymatic reaction mixtures that offer improved efficiencies in connection with the labor intensive protocols for reaction mixture formulation, and reaction product characterization.

The invention is thus generally directed to the provision of a composition comprising an essential component of an enzymatic reaction combined with a tracer which is compatible with the enzyme, where the composition contains an essential absence of the substrate. The composition can have a density at least about 1.01 g/cm^3. This composition is particularly useful for any enzyme reaction where post-reaction processing or analysis is benefited by the tracer and/or increased density of the reaction mixture. In particular, such compositions for polymerase and restriction enzyme reactions are provided, where the presence of the tracer and/or increased density is useful for post-reaction electrophoretic analysis. Methods for using these compositions, and methods for preparing these compositions are also provided.

The invention is directed, therefore, to a composition which is suitable for formulation of an enzymatic reaction mixture, the composition comprising a reaction component essential for an ex-vivo non-polymerase enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, and a tracer compatible with the enzyme, wherein the composition is substantially free, or has an essential absence, of the substrate. These compositions

can further comprise a density of at least about 1.01 g/cm^3.

The present invention is also directed toward a composition comprising a reaction component essential for an ex-vivo enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product and a tracer compatible with the enzyme, the composition being substantially free or having an essential absence of the substrate and having an optical density greater than about 5 at a visible wavelength of maximal tracer absorbance.

The present invention is further directed toward a composition comprising a reaction component essential for an ex-vivo polymerase reaction in which a nucleic acid polymer product complementary to a nucleic acid polymer template is prepared, and a tracer compatible with the polymerase, the composition being substantially free or having an essential absence of the template and has an optical density greater than about 5 at a visible wavelength of maximal tracer absorbance. These compositions can also comprise a density of at least about 1.01 g/cm^3.

The present invention is still further directed toward a composition for an enzymatic reaction component which comprises a reaction component essential for an ex-vivo enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, and an alkaline earth-metal salt of an anionic tracer.

The present invention is also directed toward a composition which comprises a reaction component essential for an ex-vivo enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, and a tracer selected from the group consisting of acid red 106, acid red 4, acid red 1, amaranth, and acid violet 5, or a salt thereof.

The present invention is further directed toward a composition comprising a reaction component essential for an ex-vivo enzymatic reaction in which a nucleic acid polymer substrate is enzymatically cleaved by a restriction enzyme in a reaction mixture to form a restriction product, and a tracer compatible with the restriction enzyme, wherein the composition contains an essential absence of the substrate. These compositions can also comprise a density of at least about 1.01 g/cm^3.

The present invention is still further directed toward an improvement in a method for a polymerase reaction that comprises forming a reaction mixture comprising a polymerase, a nucleic acid polymer template, a tracer compatible with the polymerase, and other components essential for the polymerase reaction, creating a nucleic acid polymer product complementary to the nucleic acid by enzymatic reaction, analyzing the product of the enzymatic reaction by an electrophoretic protocol, and observing the tracer during the electrophoretic protocol without providing additional tracer beyond that which was included in the reaction mixture. The improvement comprises supplying the tracer to the reaction mixture in a composition that comprises the tracer and the enzyme or another essential component, the composition being substantially free or having an essential absence of the nucleic acid polymer template. A further improvement is in the reaction mixture having a density at least about 1.01 g/cm^3.

The present invention is further directed toward an improvement in a method for a polymerase reaction that comprises forming a reaction mixture comprising a polymerase, a nucleic acid polymer template, a tracer compatible with the polymerase, and other components essential for the polymerase reaction, creating a nucleic acid polymer product complementary to the nucleic acid by

enzymatic reaction, analyzing the product of the enzymatic reaction by an electrophoretic protocol, and observing the tracer during the electrophoretic protocol. The improvement comprises supplying the tracer to the reaction mixture in a composition that comprises the tracer and the enzyme or another essential component, the composition being substantially free or having an essential absence of the nucleic acid polymer template, wherein the tracer supplied to the reaction mixture is of adequate character and sufficient quantity to be visible during the electrophoretic protocol.

The present invention is also directed toward a method for a restriction enzyme reaction, the method comprising forming a reaction mixture comprising a restriction enzyme, a nucleic acid polymer substrate, a tracer compatible with the restriction enzyme, and other components essential for the enzymatic reaction, enzymatically cleaving the nucleic acid polymer substrate to form a restriction product, analyzing the product of the cleavage reaction by an electrophoretic protocol, and observing the tracer during the electrophoretic protocol without providing additional tracer beyond that which was included in the reaction mixture. The density of the reaction mixture can also be at least about 0.01 g/cm greater than the liquid phase utilized in the chromatographic or electrophoretic protocol.

The present invention is also directed toward a method for a restriction enzyme reaction, the method comprising forming a reaction mixture comprising a restriction enzyme, a nucleic acid polymer substrate, a tracer compatible with the restriction enzyme, and other components essential for the enzymatic reaction, enzymatically cleaving the nucleic acid polymer substrate to form a restriction product, analyzing the product of the cleavage reaction by an electrophoretic protocol,

wherein the tracer supplied to the reaction mixture is of adequate character and sufficient quantity to be visible during the electrophoretic protocol.

The present invention is further directed toward a method for forming an enzymatic composition, the method comprising combining a reaction component with a tracer, the reaction component being essential for an enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, the tracer being compatible with the enzyme, and the resulting composition having an optical density greater than about 15 at a visible wavelength of maximal tracer absorbance. A liquid which is compatible with the enzyme can also be added, wherein the liquid increases the density of the composition to at least about 1.1 g/cm^3.

The present invention is still further directed toward a method for forming an enzymatic composition, the method comprising combining a reaction component with a tracer, the reaction component being essential for a polymerase reaction in which a nucleic acid product is polymerized from a complementary nucleic acid template, the tracer being compatible with the enzyme, and the resulting composition having an optical density greater than about 5 at a visible wavelength of maximal tracer absorbance. A liquid which is compatible with the enzyme can also be added, wherein the liquid increases the density of the composition to at least about 1.1 g/cm^3.

Other features, objects and advantages of the present invention will be in part apparent to those skilled in the art and in part pointed out hereinafter. All references cited in the instant specification are incorporated by reference. Moreover, as the patent and non-patent literature relating to the subject matter disclosed and/or claimed herein is substantial, many relevant references are available to a skilled artisan

that will provide further instruction with respect to such subject matter.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides compositions comprising an essential component to an enzyme reaction and a tracer which can allow post-reaction analysis without further tracer addition. These compositions ("analysis reagent compositions") can also comprise a high density agent which can eliminate the need for further addition of a high density agent during post-reaction analysis. Methods of using these compositions, and methods of preparing them are also provided.

Prior art methods of using loading buffer components always added these components to the reaction mixture, either before or after the enzymatic reactions are executed. Such methods require an extra step to add the loading buffer components. An improvement over the prior art in the present invention is the provision of analysis reagent compositions comprising an essential component for an enzymatic reaction combined with the loading buffer components. As diagrammed in Figure 3, the approach of the present invention offers further efficiencies that were not achieved with prior art protocols. The loading buffer components are passively added with the essential component, thus eliminating the extra addition step required in the prior art to add the loading buffer. In laboratories where many enzyme reaction mixtures are prepared, the compositions and methods of the present invention can thus eliminate a considerable amount of work. These analysis reagent compositions are particularly useful for molecular biological enzyme reactions, particularly polymerase reactions and restriction enzyme reactions, since these

reactions are often repetitively performed under standardized conditions.

The procedures disclosed herein which involve the molecular manipulation of nucleic acids are known to those skilled in the art. *See generally* Joseph Sambrook et al. (1989), "Molecular Cloning, A Laboratory Manual", second ed., Cold Spring Harbor Laboratory Press.

As used herein, the term "substrate" encompasses a component of an enzymatic reaction mixture which is a reactant in the reaction catalyzed by the enzyme. For example, in a restriction enzyme reaction, the substrate is the nucleic acid polymer which is cut by the enzyme. In a polymerase chain reaction, the substrates include the primer which is extended by the enzyme, and the nucleotides which are added to the growing nucleic acid polymer.

The present invention provides compositions comprising a reagent which is essential for an enzymatic reaction ("essential reagent") and a tracer which is compatible with the enzyme. A high density agent may also be included in these compositions. Methods are also provided wherein the compositions are used in an enzymatic reaction and the results are subsequently analyzed by electrophoresis or chromatography in a water soluble solvent.

The compositions and methods of the present invention are useful in conjunction with any enzymatic reaction where the reaction products are subsequently analyzed by a chromatographic or electrophoretic method. Enzymes which can be employed include those which modify or degrade proteins, lipids, carbohydrates, and metabolites, such as any kinase, protease, lipase, amylase, peroxidase, oxidase, oxygenase, and dehydrogenase. Enzymes which modify, cut, or synthesize nucleic acids are particularly suitable to be used with

the present invention. Examples include any ligase, phosphodiesterase, DNase, exonuclease, RNase, phosphatase, kinase, terminal transferase, reverse transcriptase, restriction endonuclease, RNA polymerase, and DNA polymerase. Enzymes which are preferred for use with this invention are restriction endonucleases and DNA polymerases. More preferred are DNA polymerases; even more preferred are any thermostable DNA polymerase; most preferred is wild-type or modified Taq polymerase. These enzymes can be in any concentration which is useful for performing an enzymatic reaction. Preferred concentrations of Taq polymerase in the compositions of the present invention are 0.033 - 10 units/μl, more preferred concentrations are 0.06 - 5 units/μl, the most preferred concentration is 1 unit/μl. Preferred concentrations of restriction endonucleases in the compositions of the present invention are 0.1 - 1000 units/μl, more preferred concentrations are 1 - 100 units/μl, most preferred concentrations are 5 - 40 units/μl.

Tracers which can be used in this invention include detectable compounds which can be incorporated into the reaction mixture and not interfere significantly with the enzyme reaction. Such a tracer is designated herein as "compatible" with the enzyme. It is preferred that this compatibility be such that an enzyme composition with the tracer has at least 95% of the activity of the same composition without the tracer. More preferably, the tracer-enzyme composition has at least about 97% activity of the composition without the tracer, even more preferably at least about 99% activity, and most preferably about 100% activity. The tracer should also be stable enough in the tracer-enzyme composition to retain its compatibility with the enzyme even after a

long storage period at an appropriate temperature, e.g. 1 year or more at -20°C.

The detectable signal imparted by the tracer can be visual, such as that imparted by a dye or fluorescent compound. The tracer can also impart a radioactive, electrochemical, spectrophotometric, or any other type of signal which can be detected sensually or with an instrument and which can serve as a useful marker in an analysis subsequent to the enzyme reaction. Preferred are tracers which impart a visual signal. The most preferred tracers are dyes which are colored under the conditions that the analysis is performed. Any color dye which is visible during the post-reaction analysis can be used; preferred are dyes which have a peak visible absorbance wavelength at between 430 and 617 nm; most preferable dyes have a peak visible absorbance wavelength at between 500 and 535 nm.

While any tracer compatible with the enzyme can be useful in the present invention, preferred tracers are highly soluble in the liquid phase of the post-reaction chromatographic or electrophoretic procedure. The tracer is preferably an anionic tracer. Particularly preferred tracers are anionic tracers such as salts of organic acid dyes or sulfonic acid dyes. The preferred salt counterion is an earth metal, most preferably Ca^{++} or Mg^{++}. Where the post-reaction analysis is an electrophoresis of a nucleic acid, preferred tracers are anionic dyes. Preferred concentrations of the tracer in an analysis reagent composition are concentrations for which the composition has an optical density (OD) of between about 5 and about 500; most preferred is about 300. In enzyme reaction mixtures prepared from the analysis tracer composition the preferred tracer concentration has an OD of between 1 and 100; more preferred is 15 to 50; most preferred is 15.

Commercial preparations of tracers are often inhibitory to enzyme activity when used in the concentrations recited above. This inhibition can often be overcome by further purification of the tracer, for example by reverse phase desalting, recrystalization, acid precipitation, or chromatographic methods such as reverse phase, normal phase, or ion exchange chromatography. Where the tracer is an anion, such as disclosed in Example 1 below, enzyme inhibition can also be overcome by replacing the counterion with an alkaline earth-metal. Preferred alkaline earth-metals for this purpose are Ca^{++} and Mg^{++}; most preferred is Mg^{++}.

High density agents useful for the present invention include any solute in which the tracer is soluble and which is compatible with the enzyme when diluted in the final reaction mixture, and which is dense enough to assist in the addition of reaction mixture to the analytical process. To provide such assistance, the density of the reaction mixture should be at least about 0.01 g/cm^3 greater than the density of the liquid phase of the analytical process (e.g. the electrophoresis or chromatographic buffer). A somewhat higher density (for example, about 0.05 g/cm^3 greater than the analytical liquid phase) would provide greater assistance in the addition of the reaction mixture, and is thus more preferred. These densities may be provided using the preferred density of an essential enzyme component/tracer/high density agent composition of about 1.14 g/cm^3. Higher densities are also useful, however, provided they are compatible with the enzyme at the concentration used in the enzyme reaction.

Examples of solutes which are generally compatible with enzymes at the concentrations required to provide sufficient density are sucrose or other sugars, glycerol, and betaine (trimethylglycine). Glycerol is preferred.

Glycerol at a concentration of 1.5% in water is about 0.01 g/cm^3 more dense than water, and would thus provide assistance in applying a sample to an analytical process where water is the liquid phase. Glycerol at a concentration of 50% in water has a density of about 1.14 g/cm^3. Thus, 50% glycerol is preferred as the high density agent in an essential enzyme component/tracer/ high density agent composition.

A scheme which is useful for evaluating the compatibility and effectiveness of a tracer and/or high density agent ("loading buffer component") for this invention is shown in Fig. 4. In this scheme, the desired physical characteristics of the loading buffer component is determined. For example, the desired color and charge of a dye to be used as a tracer is decided. Next, a set of candidates (e.g. red, anionic dyes) is assembled for testing to evaluate other desired properties (e.g. enzyme compatibility, lack of interference in genetic transformation protocols, etc). The candidates are then tested for these desired properties, preferably by performing the least laborious tests first, in order for the largest amount of undesirable candidates to be eliminated by the least amount of screening. When desirable loading buffer components are selected, the formulation of loading buffer component and essential reagent is prepared, and its effect on the enzyme reaction and subsequent analysis is characterized.

Any essential reagent can be selected to be combined with the loading buffer component to formulate a composition of the present invention. The selection of an essential reagent for this purpose can depend on factors such as:

- whether it is desired to be able to determine if the essential reagent has been added to the enzyme reaction

mixture. For example, if the essential reagent is an enzyme and the loading buffer component is a dye, then one can easily determine if the enzyme has been added to the reaction mixture by determining if the reaction mixture is colored.

- whether the essential reagent might be added at varying concentrations in several reaction mixtures, or whether there are alternative formulations of essential reagents which might be added. For example, a particular buffer solution can be used at different concentrations by several different restriction endonucleases. *See* Joseph Sambrook et al. (1989), "Molecular Cloning, A Laboratory Manual", second ed., Cold Spring Harbor Laboratory Press, at pp. 5.28-5.31. The concentrated buffer solution may not be a preferred essential reagent to combine with the loading buffer components under those conditions, since the final reaction mixtures could have varying concentrations of the loading buffer components, depending on the enzyme used. However, where the concentrated buffer solution is generally always diluted a particular amount, such as with 10X buffers which are often provided for particular enzymes (e.g. restriction enzymes), the loading buffer components can be usefully provided in combination with these solutions.

Examples of essential reagents which can be combined with loading buffer components to formulate a composition of the present invention are: enzyme, concentrated enzyme buffer (e.g. 10X buffer), a nucleotide or primer reagent in the case of DNA or RNA polymerases, or a coenzyme such as NADPH or ATP. The preferred essential agent for this purpose is the enzyme, since it is often desirable to be able to ascertain if enzyme addition has taken place, and since enzyme concentrations in reaction mixtures are generally not widely varied. A colored enzyme formulation also has the advantage of allowing one to

determine if complete mixing of the enzyme has taken place. If the solution is uniformly colored then the enzyme is uniformly distributed. Also, since a colored formulation is more readily visible than a clear formulation, a colored enzyme formulation also facilitates pipetting of the small volumes of enzyme which are often added to enzymatic reaction mixtures.

Inclusion of loading buffer components with the substrate is usually not preferred because the substrate composition and concentration often varies between individual enzyme reactions. For a PCR reaction, however, the loading buffer components can be advantageously added with the nucleotide substrates, since the concentration of these reagents generally do not vary between individual PCR reactions.

As contemplated by the present invention, the analysis of the product of an enzyme reaction can be by any method which is suitable for the product in question. Chromatographic and electrophoretic methods are particularly suitable. Suitable chromatographic methods include liquid chromatography ("LC"), particularly gel permeation chromatography. In LC, a high density agent would facilitate the loading of the reaction mixture containing the product onto a chromatographic column, and a visible tracer would allow one to follow the progression of the sample through the column.

For applications where the product to be analyzed is a nucleic acid polymer, electrophoretic methods are preferred. In this regard, the compositions and methods of the present invention are useful for agarose gel electrophoresis (e.g. to analyze products of PCR, restriction endonuclease digestion, ligation reactions, etc), and polyacrylamide gel electrophoresis (e.g. analysis of sequencing reactions). Polyacrylamide gel electrophoresis is also facilitated by the present

invention when used to analyze protein products of enzymatic reactions.

The following examples illustrate the invention, but are not to be taken as limiting the various aspects of the invention so illustrated.

Example 1. Identification and formulation of a Taq DNA polymerase with tracers and high density reagent

A composition conforming to the present invention was developed to facilitate analysis of products resulting from PCR. The composition comprises an essential PCR component, Taq DNA polymerase, with sufficient glycerol to facilitate the application of the reaction product to an agarose gel for electrophoretic analysis. The composition also comprises a red dye that aids in visualization and mixing of the enzyme in the reaction mixture. The red dye also serves as a tracer to follow the progressive movement of PCR products through an agarose gel during electrophoresis. The color red was selected for aesthetic reasons and confers no particular advantage as a tracer.

Fig. 5 summarizes the steps taken to develop this composition. Since nucleic acid products of PCR are highly anionic, they are applied to the agarose gel near the anode and move toward the cathode as the electrophoresis progresses. Therefore, to be useful as a tracer in electrophoresis the dye molecule is preferably anionic. Figure 6 summarizes the selection process. From 180+ red dyes (absorbance max between 450 and 570 nm) (Table 1) approximately 40 anionic dyes were selected (Table 2).

Table 1

Dyes initially considered

Dye	λ_{max}
Bis-N-methylacridinium nitrate	430
4-(p-Nitrophenylazo)-resorcinol	432
Auramine O	432
Martius Yellow	432
3',3",5',5"-Tetraiodophenolsulfonephthalein	433
6'-Butoxy-2,6-diamino-3,3'-azodipyridine	435
Quinoline Yellow A, spirit soluble	435
m-Cresol Purple, sodium salt	436
Methyl Red, sodium salt	437
Methylthymol Blue, water soluble	438
a-Naphthyl Red	439
Palatine Fast Yellow BLN	440
Twort Stain	440
Pyrocatechol Violet	441
Acridine Yellow G	442
Mordant Brown 33	442
2-(5-Bromo-2-pyridylazo)-5-(dimethylamino)phenol	443
Disperse Orange 3	443
Acid Yellow 99	445
Thymolphthalein monophosphoric acid, disodium salt hydrate	445
Acid Orange 51	446
Eriochrome Cyanine R	446
Malachite Green Carbinol base	446
Ethyl Red	447
Chrysoidin	449
Orange G	475
Sudan I	476
trans-p-Carotene	478
Fast Yellow	480
Pyrogallol Red	480
Direct Black 22	481
Crocein Orange G	482
Rosolic Acid	482
Disperse Orange 1	483
Eriochrome Red B	483
Orange 11	483
Thorin I	483
Purpurin	485
Quinizarin	485
Mordant Brown 1	487
Acridine Orange	488
Para Red	488
Acridine Orange	489

Dye	λ_{max}
Acid Orange 8	490
Astrazon Orange G	490
Fluorescein diacetate	490
Fluorescein isothiocyanate, isomer I	490
Quinalizarin	490
Tropaeolin 0	490
Zincon	490
Zincon, monosodium salt	490
Fluorescein, water soluble	491
Acridine Orange hydrochloride	492
Mordant Brown 48	492
Methyl Red hydrochloride	493
Sudan 11	493
Acid Red 183	494
Reactive Orange 16	494
Carminic acid	495
Disperse Red 19	495
Fluoresceinamine, isomer 11	495
Fluorescein	496
Fluoresceinamine, isomer I	496
BrilliantYellow	497
Congo Red	497
Acid Red 97	498
Cochineal	498
Arsenazo I	499
Fluorexon	499
Benzopurpurin 4B	500
Mordant Brown 4	500
Reactive Red 8	500
Acid Alizarin Violet N	501
Rhodamine 123 dehydrate	501
Darrow Red	502
Disperse Red 1	503
Xylidine Ponceau 3RS	503
Acid Red 106	505
Acid Red 88	505
Biebrich Scarlet, water soluble	505
Nuclear Fast Red	505
Acid Red 4	506
New Coccine	506
2-(4-Sulfophenylazo)-1,8-dihydroxy-3,6-naphthalene-disulfonic	507
Direct Red 23	507
Merbromin	507
Methyl Orange	507
Sudan III	507
Toluidine Red	507

Dye	λ_{max}
Acid Red 4	508
Acid Red 8	508
Direct Red 81	508
2',7'-Dichlorofluorescein	509
Brilliant Crocein MOO	510
Chromotrope 2R	510
Basic Red 29	511
Acid Red 151	512
Chromoxane Cyanine R	512
Quinalizarin	512
Acid Red 37	513
Acid Red 114	514
Chromotrope 2B	514
Eosin B	514
Eosin Y	514
Ponceau SS	514
Acid Red 150	515
Chromotrope FB	515
Acid Red 40	516
Azocarmine B	516
Mordant Blue 9	516
Reactive Red 4	516
Cibacron Brilliant Red 3B-A	517
Disperse Red 13	517
Eosin Bluish blend	517
4,5,6,7-Tetrachlorofluorescein	518
Bordeaux R	518
Oil Red 0	518
Acid Violet 7	520
Methyl eosin	520
Ponceau S	520
Rose Bengal, bis(triethylammonium) salt	520
Sudan IV	520
Amaranth	521
Emodin	521
Eosin Y, free acid	521
Giemsa Stain	521
Oil Red EGN	521
Purpuri'n	521
Azure A eosinate	522
Diiodofluorescein	522
Direct Red 75	522
Eosin B, spirit soluble	522
Jenner Stain	522
Leishman Stain	522
May-Grbnwald Stain	522
Wright Stain	522

Dye	λ_{max}
Wright Stain, solution in methanol	522
Azure B eosinate	523
Zincon, monosodium salt	523
Acid Blue 120	524
Azure 11 eosinate	524
Eosin Y lactone	524
Rhodamine 6G	524
Tetrachrome Stain (MacNeal)	524
Erythrosin B	525
Erythrosin Yellowish blend	525
Ethidium bromide	525
Acid Violet 5	527
Plasmocorinth B	527
Eriochrome Blue Black 2B	528
Quinaldine Red	528
Rhodamine 6G Perchlorate	528
Rhodamine 6G tetrafluoroborate	528
Sulforhodamine G	529
Violamine R	529
Chromotrope 2R	530
Safranine 0 (Y,T)	530
Alum Carmine	531
Carmine	531
Acid Red 1	532
Acid Red 106	532
Ethyl Eosin	532
Arsenazo 111, sodium salt hydrate	533
Erythrosin B, spirit soluble	533
Sudan Red 7B	533
Ruthenium Red	534
Nuclear Fast Red	535
Acid Red 40	538
Alizarin Violet 3R	540
Neutral Red	540
Aluminon	542
Rhodamine B	543
Basic Fuchsin	544
Basic Fuchsin, special for flagella	544
Pararosaniline base	544
Rhodamine B base	544
Acid Fuchsin, calcium salt	545
Acid Violet 17	545
Aurintricarboxylic acid	545
Aurintricarboxylic acid, trisodium salt	545
Pararosaniline acetate	545
Acid Fuchsin, sodium salt	546
Carbol Fuchsin	547

Dye	λ_{max}
Alizarin Blue Black B	548
Phloxine B	548
Pyronin Y	548
Rose Bengal	548
Basic Fuchsin, biological stain	549
Direct Violet 51	549
9-Phenyl-2,3,7-trihydroxy-6-fluorone	552
Bromopyrogallol Red	552
Phenolphthalein	552
Rhodanile Blue	552
New Fuchsin	553
Nile Red	553
Pyronin B	553
Sulforhodamine B	554
Alizarin Red S monohydrate	556
Methylene Violet 3RAX	557
PhenolRed	557
Rose Bengal, bis(triethylammonium) salt	559
Arsenazo III	560
Pinacyanol chloride	560
Acid Blue 161	563
Carmine	563
Nigrosin, alcohol soluble	565
Acid Blue 113	566
o-Cresolphthalein	566
Alizarin	567
Sulfonazo 111, tetrasodium salt	567
Palatine Chrome Black 6BN	569
Brilliant Black BN	570
Cresol Red	570
Bromocresol Green (also broad absorbance at 417 nm)	617

Table 2

Properties of anionic dyes

No	Dye	λ_{max} (nm)	Color	EtOH	Qiagen	PCR
1	Orange G	475	X			
2	Acid Red 150	515		X		
3	Acid Red 88	505	solubility			
4	Acid Red 106	532			X	
5	m-Cresol Purple, sodium salt	436	X			
6	2-(4-Sulfophenylazo)-1,8-dihydroxy-3,6-naphthalene-disulfonic acid, trisodium salt	507			X	
7	Mordant Blue 9	516	X			
8	Chromotrope 2R	510				X
9	Pyrogallol Red	480				X
10	Reactive Red 4	516				X
11	Disperse Orange 1	483	X			
12	Congo Red	497	X			
13	Direct Red 81	508		X		
14	Phloxine B	548	X			
15	Eriochrome Cyanine R	446	X			
16	Acid Violet 17	545	X			
17	Chromotrope 2B	514			X	
18	Zincon, monosodium salt	490	X			
19	Methyl Red, sodium salt	437	X			
20	Acid Orange 8	490	X			
21	Rosolic Acid	482	solubility			
22	Eosin Y	514	X			
23	Bordeaux R	518				X
24	Acid Red 106	505				
25	Acid Red 4	506				
26	Acid Red 1	532				
27	Bromocresol Green (also broad absorbance at 417 nm)	617	X			
28	Ponceau S	520		X		
29	Benzopurpurin 4B	500	solubility			
30	Acid Orange 51	446	X			
31	Amaranth	521				
32	4-(p-Nitrophenylazo0-resorcinol	432	X			
33	Biebrich Scarlet, water soluble	505		X		
34	Martius Yellow	432	X			
35	Reactive Orange 16	494				X
36	Direct Violet 51	549	X			
37	Chromotrope FB	515			X	
38	Direct Red 75	522		X		
39	Acid Violet 5	527				
40	Acid Red 97	498				X

Each of these dyes were dissolved in water and those that were not particularly red (i.e. were too yellow/orange or purple), or lacked sufficient solubility, were removed from consideration (Table 2, those marked under "Color").

The 20 dyes which remained were assayed for their ability to be removed from DNA by ethanol precipitation. To 1 μg of lambda DNA enough dye was added to yield a highly colored solution. Addition of two volumes of 3M ammonium acetate, then 6 volumes of ethanol followed. The DNA was pelleted by centrifugation. The appearance of a colored pellet caused the dye to be removed from consideration (Figure 6, EtOH ppt.; Table 2, those marked under "EtOH"). In the presence of the remaining dyes, one μg quantities of DNA were purified by solid phase extraction on Qiagen PCR product purification columns (Qiagen, Hilden, Germany) according to the manufacturers protocol. Dyes that yielded colored eluants were dropped from consideration (Figure 6, labeled "Qiagen", Table 2, those marked under column labeled "Qiagen"). The assays performed to this point were done at the onset of the screening because they were the least laborious.

The dyes that survived these preceding tests were included in a PCR toxicity study. The dyes were added to PCR reactions at concentrations considered adequate for product performance. Enough of a 2X PCR master mix consisting of Taq DNA polymerase, 0.1 u/μl (Sigma Chemical Co., St. Louis, MO), PCR buffer (2X) (Sigma), dNTP's (200 μM each) (Sigma), α^{32}PdCTP (Amersham USA, Piscataway, NJ), target DNA (lambda, 2 ng/μl) (Sigma) and primers (Perkin-Elmer 500 bp control, 2 μM) (Perkin-Elmer, Norwalk, CT) was prepared to accommodate all 11 remaining dyes plus three no dye controls. Ten μl of the 2X master mix was dispensed into reaction tubes followed by addition of 10 μl of aqueous darkly colored dye solutions or water (controls). The PCR cycling protocol

was 20 cycles of 94/55/72°C at one minute each. 20 μl of quench solution (50 μg/μl calf thymus DNA, 20 mM EDTA) was added followed by precipitation with 40 μl 40% trichloroacetic acid (TCA)/4% sodium pyrophosphate (NaPPi). The reactions were filtered on glass fiber filters, washed with 5% TCA/2% NaPPi and counted by scintillation methods. This quench/precipitation procedure will henceforth be referred to as "TCA precipitation". As shown in Figure 7, some dyes significantly inhibited the PCR reaction such that little or no product resulted (i.e. numbers 8, 9, 10, 35 and 40). However, other dyes were relatively inert (i.e. 23, 24, 25, 26, 31, 39). The dyes that inhibited PCR were dropped from consideration (Fig.6, PCR Tox, Table 2, those marked under "PCR"). Number 23 was dropped because its per cycle yield was substantially lower than the other dyes (Fig.7). Per cycle yield was calculated assuming the overall yield was the per cycle yield raised to the 20^{th} power (Figure 7).

The dyes which remained after the PCR toxicity screen were further screened for their toxicity on ligation and transformation, two downstream procedures often carried out using unpurified PCR products. As shown in Figure 8, ligation in the presence of the remaining dyes (24, 25, 26, 31 and 39 lanes 3-7 respectively) was equivalent to the no dye control (lane 2). Lane 1 is a control which had no ligase. Ligations were carried out as described in the figure legend. The same dyes were also tested for suitability in a ligation/transformation protocol. Transformation efficiency was not compromised by the presence of dye (Figure 9). Increased efficiencies, as evidenced in the figure, were not investigated. The experiment was performed as summarized in the figure legend.

On the basis of performance to this point, the remaining dyes were considered substantially equivalent as tracer candidates. Acid violet 5 (number 39, Table 2) was chosen as a possible finalist candidate. Using this dye in a reaction mix at an absorbance of 10 (at its absorbance maximum of 527 nm) a PCR reaction was performed. The inclusion of this dye in a reaction mix resulted in a minimum of 50% loss (relative to the same reaction without the dye) in PCR product yield (Figure 10) as measured by TCA precipitation. This was considered unacceptable, but was overcome by further development efforts.

In general, the available dyes, as well as those investigated, are derived from unrelated applications. That is, many are used as fabric dyes, food colorings, biological stains etc. As such, they are available in various states of purity. In the case of the finalists, they all had purities of 80% or less, as determined by the product labels. In further experiments, PCR yields (determined by TCA precipitation) in reactions containing various concentrations of acid violet 5 (determined by A_{527}) were compared before and after purification of the crude dye by reverse phase desalting. PCR product yield was much less susceptible to dye concentration for the desalted compound relative to the crude compound (Figure 11). Since reversed phase desalting is not an attractive method for large scale dye purification, acid precipitation followed by ammonium hydroxide dissolution/ evaporation to produce the ammonium dye was investigated. This procedure should result in an essentially salt free product. These procedures (reversed phase desalting and acid/ammonium hydroxide) were carried out for acid red 1 (No. 26, Table 2) and acid violet 5 (No. 39). It was found that the ammonium dyes were more PCR toxic than the sodium (i.e. desalted) dyes (Figure 12a). The dyes were

converted to their magnesium salts to further characterize the effect of counterion identity. This was accomplished by addition of magnesium chloride (excess) to a solution of the crude dye. The magnesium dye that immediately precipitated was recrystallized from water. As shown in Figure 12a, the Mg salts of both acid red 1 and acid violet 5 was much less toxic to PCR than either the sodium or ammonium salts. In analogy with the magnesium salts, the calcium and zinc salts of acid red 1 were prepared to investigate whether the effect was divalent vs. monovalent cation or cation identity specific. Figure 12b demonstrates that the magnesium salt was least toxic. From these data it was concluded that the dye was likely sequestering magnesium from the PCR reaction which caused decreased product yields.

A dye formulation of the magnesium salts of 80% acid red 1 / 20% acid violet 5 was created (percentages based on absorbance at the wavelength of each dye's maximum absorbance [acid red 1 = 531 nm, acid violet 5 = 528 nm]). That particular ratio was used for aesthetic reasons, however any ratio, or either of the dyes individually would be similarly effective. To further investigate the effects of Mg^{++}, since the dye is supplying magnesium to a magnesium dependent reaction (i.e. PCR), free Mg^{+2} concentration contributed by the dye to the reaction was determined. This was determined by varying the magnesium concentration in dye containing vs. dye free reactions in a dose response manner. Figure 13 shows that for products ranging from 500 to 3000 bp, the difference between red and white Taq at the midpoint of the magnesium concentration titrations is approximately 0.4 mM (0.37 +/-0.04). The 10X buffer usually supplied with Taq was reformulated to account for this perturbation (i.e. the concentration of $MgCl_2$ was changed from 15 to 11 mM in the 10X buffer).

A preferred composition was formulated at 1 u/μl Taq polymerase in Taq storage buffer (consisting of 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Igepal® CA-630, 50% glycerol in water) with the magnesium formulation of dye at a total absorbance of 300. The dye composition was 80% acid red 1, 20% acid violet 5 (100%= absorbance of acid red 1 at λ_{max} + absorbance of acid violet 5 at λ_{max}, absorbance of acid red 1=240, acid violet 5=60). This formulation is designated "*REDTaq*™". When added to a PCR reaction mixture at 0.05 u/μl Taq, the total dye absorbance is 15. The dye combination at this concentration was visible in a subsequent agarose gel electrophoresis of the completed reaction mix, yet the combination was relatively non-toxic to PCR. A lower concentration of the dye in the reaction mixture would be difficult to see during a subsequent agarose gel electrophoresis. As a comparison, the previously discussed prior art Taq-dye formulation, Red Hot DNA Polymerase, has an absorbance of 3.3 at 572 nm, and 4.6 at 435 nm. At the recommended concentration in a PCR reaction mixture, Red Hot DNA Polymerase has an absorbance of 0.033 and 0.046, at 572 and 435 nm, respectively. Therefore, in contrast to *REDTaq*™, the Red Hot DNA Polymerase formulation would not be useful as a tracer in an electrophoretic analysis of a PCR reaction.

PCR products prepared using the *REDTaq*™ formulation with the 10X buffer described above (with 11 mM $MgCl_2$) were compared with conventional Taq/10X buffer (with 15 mM $MgCl_2$). Figure 14 shows a 1% agarose gel of amplification products resulting from this comparison. From the gel it is apparent that the amplifications using *REDTaq*™ were equivalent to those using Taq without dye. The exception to this is amplification of the 3 kb fragment, where the amplification with conventional Taq failed for unknown reasons (Lane 4). However, when

product yields were compared for a variety of target sizes (Figure 15), both conventional Taq and *REDTaq*™ did effectively amplify a 3 kb target. In that comparison, product yield was not compromised by *REDTaq*™. When a similar comparison was made with *Redi*Load, a commercial formulation of a red loading buffer (without an essential reaction component) which is added before a PCR reaction, the *Redi*load product reduced PCR product yield by approximately 10% (Figure 16), relative to the same reaction without *Redi*load.

Example 2. Determination of the compatibility of a dye with restriction endonucleases

The effectiveness of restriction enzymes in cutting target DNA when a dye is present in the reaction mixture was evaluated. A variety of restriction enzymes were assayed for the detrimental effect of adding Amaranth (No. 31, Table 2) to a restriction digest as assayed by agarose gel electrophoresis. Nde I-cut pUC19 plasmid was prepared. This linearized plasmid was then digested with one of several restriction enzymes which normally cut pUC19 at a polylinker site. Thus, each enzyme would be expected to yield a product of similar sizes (ranging from 212 to 263 bp). The restriction enzyme digests were performed in the presence, or absence, of Amaranth dye, and at various concentrations of the enzyme. The results of this experiment are shown in Fig. 17. Column 1 (the leftmost column) contains 100 bp molecular weight ladders. Columns 2-4 contains cleavage products from sequential 5-fold dilutions of the restriction enzymes in the presence of dye. Columns 5-7 are as columns 2-4 but without dye. Columns 2 and 5 contained the restriction enzyme at 1/10th the suppliers concentration (i.e. the enzyme was considered a 10X concentration). The buffers used for the digests were as recommended by the supplier.

The enzymes used are listed along the right side of the gel. The relative susceptibility of the enzyme to the dyes' presence is reported next to the enzyme. The sizes of the small bands in the electrophoresis runs range from 212 to 263 bp. The larger, brighter bands contain the full length NdeI-cut pUC19 (2686 bp) and/or the larger fragment of the NdeI-cut pUC19 which was also cut with the test enzyme (2423-2474 bp). +++ equals inert, blank is completely toxic. This preliminary experiment reveals that the restriction enzymes tested were relatively insensitive to dye addition. That is, with the exception of KpnI, Amaranth was relatively inert in these reactions. This experiment was conducted using commercially prepared (crude) dye (dye content approximately 90%). Based on the previously discussed results with Taq polymerase, a dye screening and possible cleanup/counterion exchange, similar to that used in Example 1, should result in the discovery of a dye system that would work for a majority of restriction enzymes.

To enable direct gel loading, the restriction digest would have to contain a component that made the solution more dense than the electrophoresis buffer. Like the PCR product, glycerol or another high density agent should suffice. Generally, star activity (alternate site cutting of DNA due to high glycerol content) can occur at glycerol concentrations above 5%. For *REDTaq*™, solutions as low as 1.5% glycerol were effective as a high density agent. Thus, it is clearly possible that restriction digest reagents could be formulated to contain enough glycerol or other density increasing solutes to allow for direct gel loading.

Other features, objects and advantages of the present invention will be apparent to those skilled in the art. The explanations and illustrations presented herein are intended to acquaint others skilled in the art

with the invention, its principles, and its practical application. Those skilled in the art may adapt and apply the invention in its numerous forms, as may be best suited to the requirements of a particular use. Accordingly, the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention.

WHAT IS CLAIMED IS:

1. A composition suitable for formulation of an enzymatic reaction mixture, the composition comprising a reaction component essential for an ex-vivo non-polymerase enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, and a tracer compatible with the enzyme, the composition being substantially free of the substrate.

2. The composition of claim 1 having a density of at least about 1.01 g/cm^3.

3. The composition of claim 1 having a density of at least about 1.1 g/cm^3.

4. The composition of claim 1 having an optical density greater than about 5 at a visible wavelength of maximal tracer absorbance.

5. The composition of claim 1 wherein the optical density of the composition is at least about 15 at a visible wavelength of maximal tracer absorbance.

6. The composition of claim 1 wherein the optical density of the composition is about 200 - 400 at a visible wavelength of maximal tracer absorbance.

7. The composition of claim 1 wherein the reaction component essential for an enzymatic reaction comprises a concentrated buffer solution.

8. The composition of claim 1 wherein the reaction component essential for an enzymatic reaction comprises an enzyme.

9. The composition of claim 8 wherein the enzyme is a nucleic acid-modifying enzyme.

10. The composition of claim 1 wherein the tracer is comprised of a dye selected from the group consisting of acid violet 5, acid red 1, and amaranth.

11. A composition comprising a reaction component essential for an ex-vivo enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, and a tracer compatible with the enzyme, the composition being substantially free of the substrate and having an optical density greater than about 5 at a visible wavelength of maximal tracer absorbance.

12. The composition of claim 11 wherein the reaction component is essential for an ex-vivo polymerase reaction in which a nucleic acid polymer product complementary to a nucleic acid polymer template is prepared, the tracer is compatible with the polymerase, and composition is substantially free of the nucleic acid polymer template.

13. The composition of claim 12 wherein the density of the composition is at least about 1.01 g/cm^3.

14. The composition of claim 12 wherein the density of the composition is at least about 1.1 g/cm^3.

15. The composition of claim 12 wherein the optical density of the composition is at least about 15 at a visible wavelength of maximal tracer absorbance.

16. The composition of claim 12 wherein the optical density of the composition is about 200 - 400 at a visible wavelength of maximal tracer absorbance.

17. The composition of claim 12 wherein the reaction component essential for a polymerase reaction is a concentrated buffer solution.

18. The composition of claim 12 wherein the reaction component essential for a polymerase reaction is a polymerase.

19. The composition of claim 18 wherein the polymerase is a thermostable polymerase.

20. The composition of claim 19 wherein the polymerase is Taq polymerase.

21. The composition of claim 12 wherein the tracer is comprised of acid violet 5 and acid red 1.

22. The composition of claim 14 wherein the optical density of the composition is about 200 - 400 at a visible wavelength of maximal tracer absorbance, the reaction component essential for a polymerase reaction is a Taq polymerase, and the tracer consists of 20% acid violet 5 and 80% acid red 1.

23. In a method for a polymerase reaction that comprises

(a) forming a reaction mixture comprising a polymerase, a nucleic acid polymer template, a tracer compatible with the polymerase, and other components essential for the polymerase reaction,

(b) creating a nucleic acid polymer product complementary to the nucleic acid polymer template by enzymatic reaction,

(c) analyzing the product of the enzymatic reaction by an electrophoretic protocol, and

(d) observing the tracer during the electrophoretic protocol without providing additional tracer beyond that which was included in the reaction mixture, the improvement comprising

supplying the tracer to the reaction mixture in a composition that comprises the tracer and the enzyme or another essential component, the composition being substantially free of the nucleic acid polymer template.

24. In the method of claim 23, the improvement further comprising the reaction mixture having an optical density at least about 15 at a visible wavelength of maximal tracer absorbance.

25. In the method of claim 23, the improvement further comprising the reaction mixture having a density at least about 1.01 g/cm^3.

26. In the method of claim 23, the improvement further comprising the tracer consisting of a combination of acid violet 5 and acid red 1.

27. In the method of claim 25, the improvement further comprising the reaction mixture having an optical density at least about 15 at a visible wavelength of maximal tracer absorbance, and the tracer consisting of a combination of 20% acid violet 5 and 80% acid red 1.

28. A method for a restriction enzyme reaction, the method comprising forming a reaction mixture comprising a

restriction enzyme, a nucleic acid polymer substrate, a tracer compatible with the restriction enzyme, and other components essential for the enzymatic reaction, enzymatically cleaving the nucleic acid polymer substrate to form a restriction product, analyzing the restriction product by an electrophoretic protocol, and observing the tracer during the electrophoretic protocol without providing additional tracer beyond that which was included in the reaction mixture.

29. The method of claim 28, wherein the optical density of the reaction mixture is at least about 15 at a visible wavelength of maximal tracer absorbance.

30. The method of claim 28, wherein the density of the reaction mixture is at least about 0.01 g/cm greater than the liquid phase utilized in the chromatographic or electrophoretic protocol.

31. The method of claim 28, wherein the tracer is amaranth dye.

32. The method of claim 29, wherein the density of the reaction mixture is at least about 0.01 g/cm greater than the liquid phase utilized in the chromatographic or electrophoretic protocol, and the tracer is amaranth dye.

33. The method of claim 28 wherein the tracer in the reaction mixture is of adequate character and sufficient quantity to be visible during the electrophoretic protocol.

ABSTRACT

A composition suitable for formulation of an enzymatic reaction mixture, the composition comprising a reaction component essential for an ex-vivo non-polymerase enzymatic reaction in which a substrate is catalyzed by an enzyme in a reaction mixture to form a product, and a tracer compatible with the enzyme, the composition being substantially free of the substrate.

Figure 1. Typical, prior art, reaction scheme.

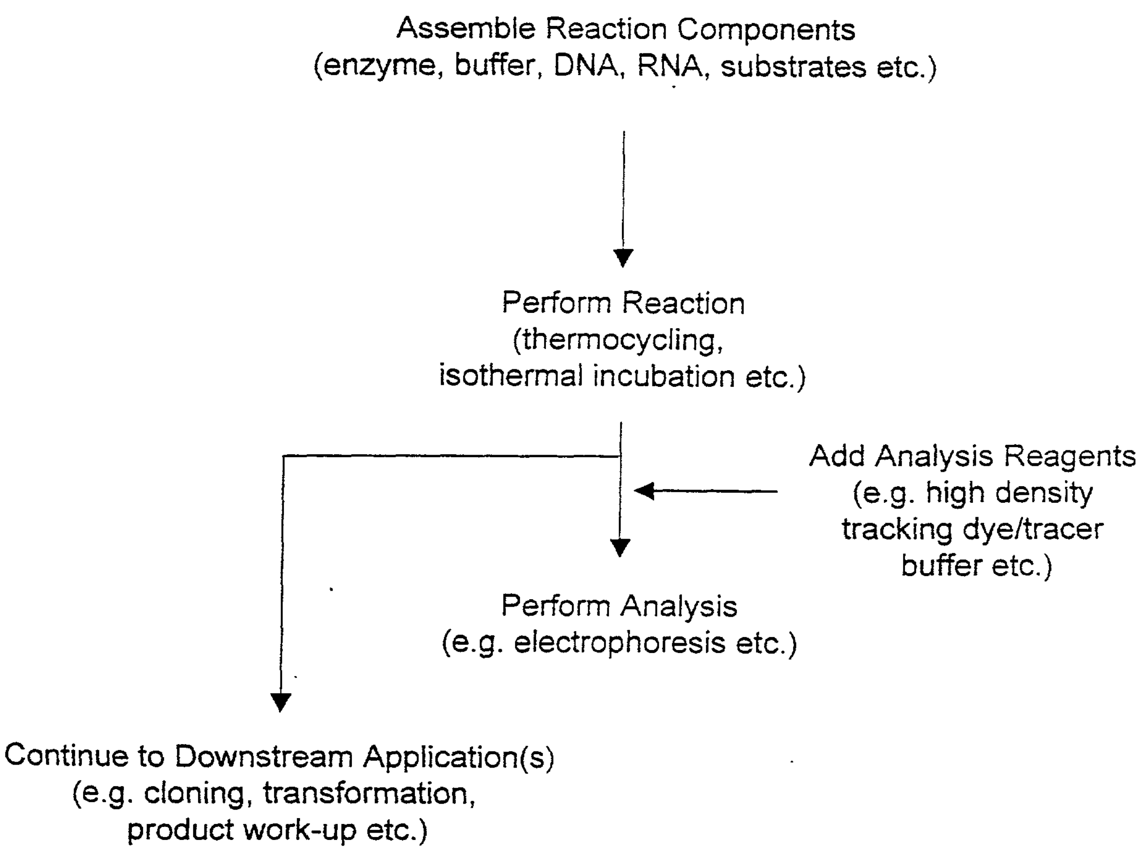


Figure 2. Modified, prior art, reaction scheme

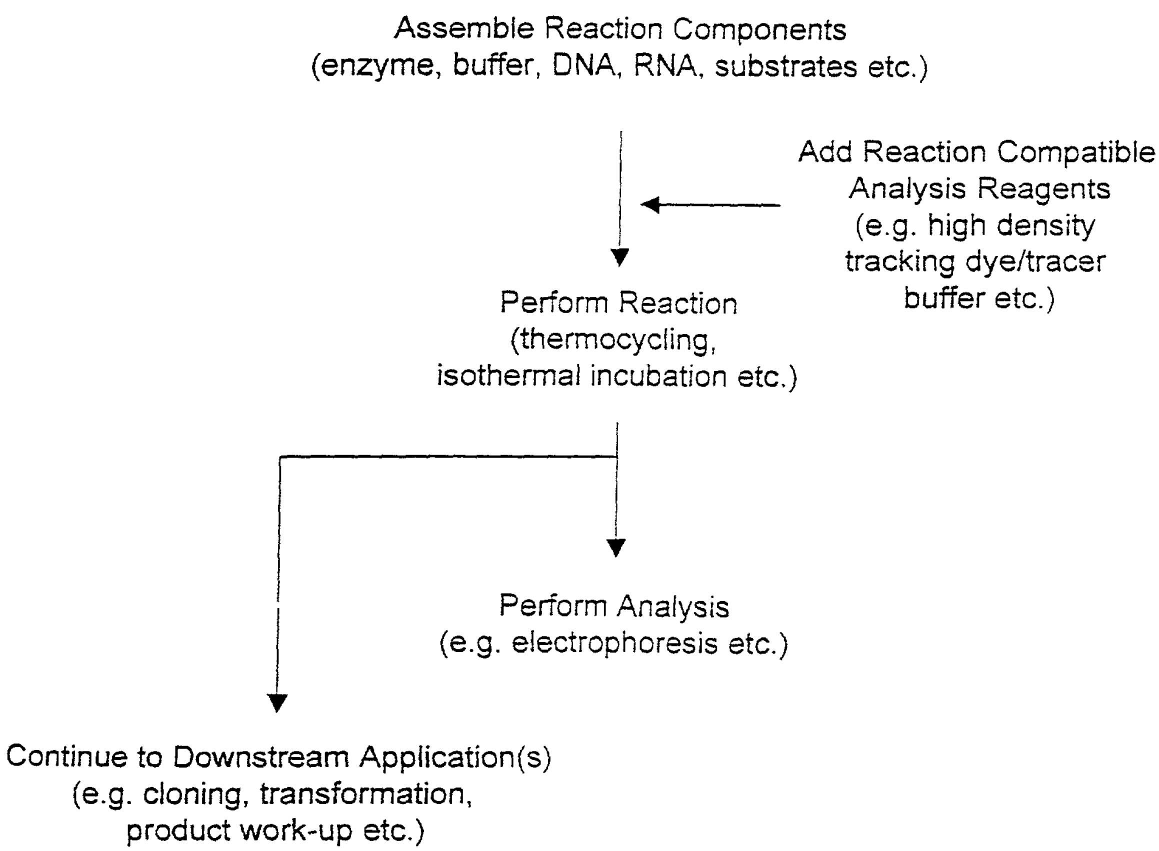


Figure 3. New reaction scheme using present invention. Any or all reaction components could be formulated to contain analysis reagents.

Assemble Reaction Components
(enzyme, buffer, DNA, RNA, substrates etc.
of which at least one has been formulated with
reaction compatible Analysis Reagents)

↓

Perform Reaction
(thermocycling,
isothermal incubation etc.)

↓

Perform Analysis
(e.g. electrophoresis etc.)

Continue to Downstream Application(s)
(e.g. cloning, transformation,
product work-up etc.)

Figure 4. Reaction compatible analysis reagent identification and optimization for PCR, sequencing, restriction digestions etc.

Physical characteristics (e.g. high density to enable gel loading, colored to visualize gel loading, anionic chromophore to track electrophoresis progress, color (if desired)).

Assemble collection of reagents for screening (e.g. collect a large sampling of anionic dyes).

Screen molecules for compatibility. The screening might be best carried out by prioritizing the desired properties from least to most laborious (e.g see red Taq Figure 5).

Formulate reagent, characterize reaction products (qualitative and quantitative). Optimize other reaction components for perturbation (if necessary). Final characterization (reaction product quality and quantity) and limitations (e.g. incompatible with specific downstream applications)

Figure 5. Red Taq development.

Physical characteristics.
High density to enable gel loading- formulate enzyme dilute enough so that enough glycerol will be contained in a 2.5 unit per 50 microliter reaction.
Colored to visualize gel loading and act as a tracking dye- red anionic water and ethanol soluble dyes were sought.

↓

Assemble collection of reagents for screening - 40 anionic "red" (lambda max =450-550) dyes were selected as candidates.

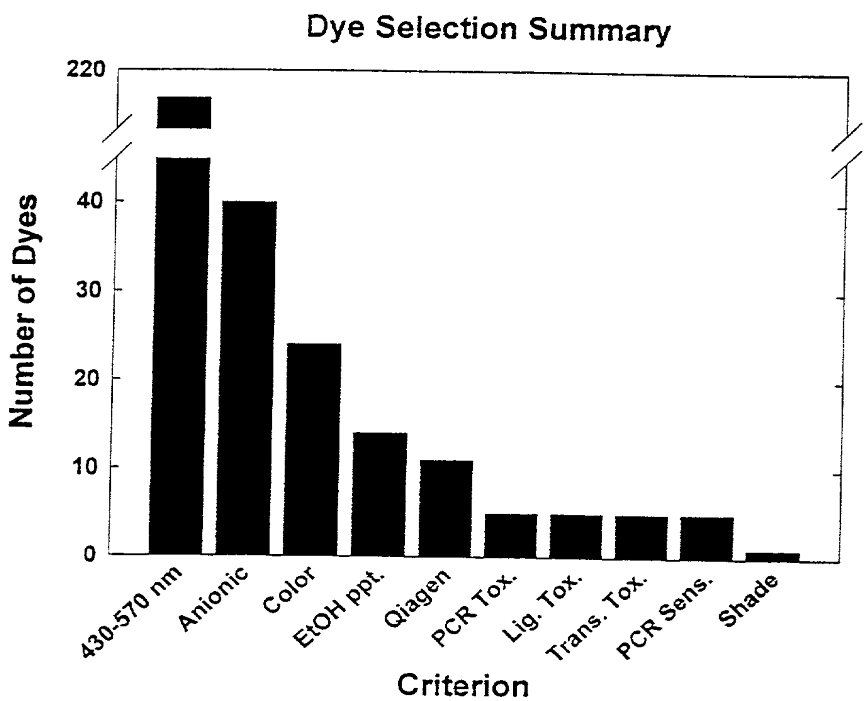
↓

Screen molecules for compatibility-summarized in Figure 6 . The dyes were scrutinized in the order:
1. Color (too yellow or purple thrown out)
2. Ethanol precipitation (colored DNA pellets thrown out)
3. Chaotropic salt/silica DNA purification (colored product thrown out)
4. PCR toxicity (Figure 7, low or no yield thrown out).
5. Ligase toxicity (Figure 8, low or no yield thrown out).
6. Transformation toxicity (Figure 9, low or no yield thrown out).
7. Remaining dyes more or less equivalent, submit to marketing for color selection.

↓

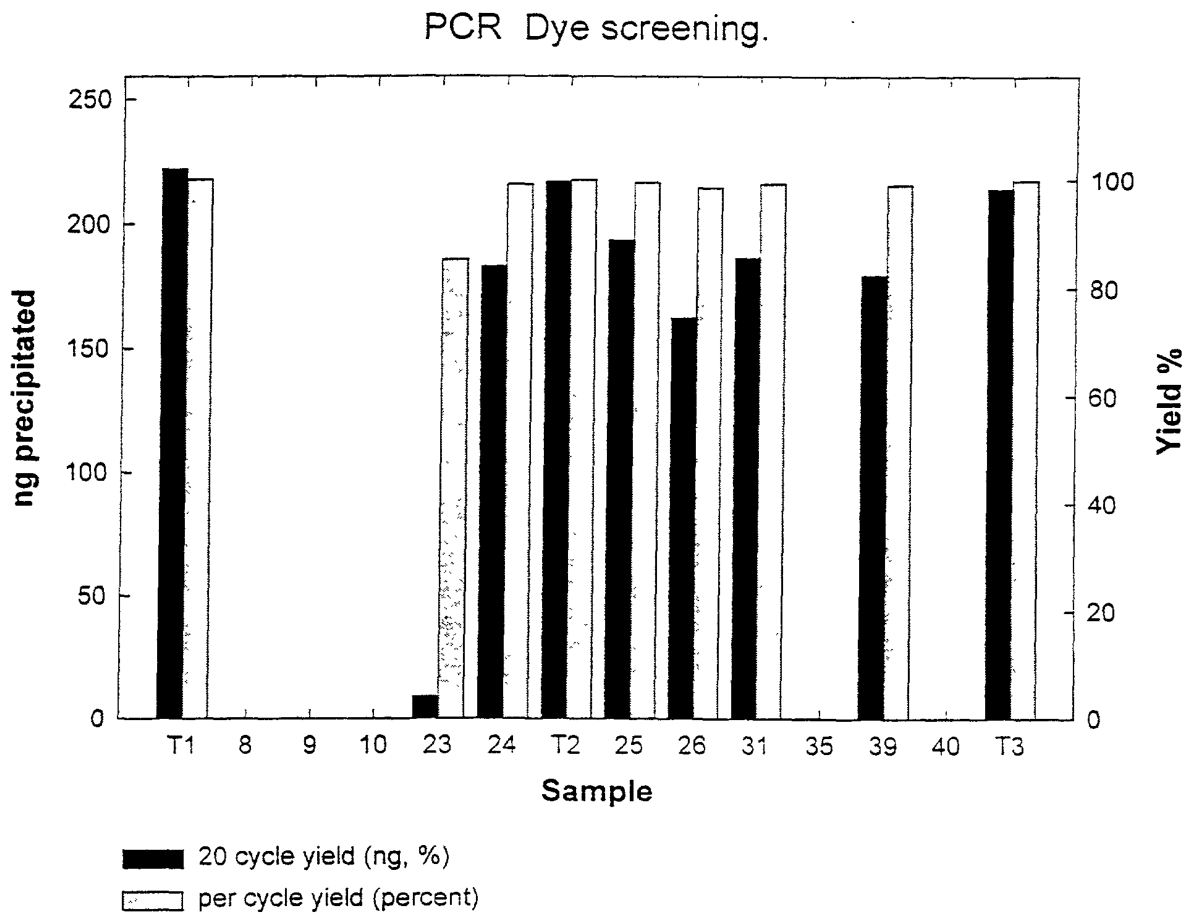
Formulate reagent- 80% acid red 1, 20% acid violet 5 (100%= absorbance of acid red 1 at lambda max + absorbance of acid violet 5 at lambda max.) to absorbance total =300 in Taq DNA polymerase at 1 unit per microliter in Taq storage buffer (20 mM Tris- HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Igepal CA- 630, 50% glycerol)
Characterize reaction products-product yields low relative to absence of dye reactions (Figure 10). Dye purity (Figure 11) and counter ion identity (Figure 12a,b) investigated for PCR toxicity/compatibility. Purified Mg acid red 1 and Mg Acid violet 5 found to be satisfactory.
Optimize other reaction components- Mg dyes contribute approximately 0.4 mM "free" Mg to PCR (Figure 13), 10X PCR buffer adjusted from 15 to 11 mM to accommodate Mg dye contribution. **Final characterization-** quality: gel (Figure 14), quantity (Figure 15). Limitations- will test with a panel of restriction enzymes, does not impact fluorescent sequencing.

Figure 6. Summary of PCR friendly dyes.



430-570 nm- visible absoprtion max.
Anionic- anionic dyes
Color-not too yellow/orange or purple
EtOH ppt.- did not co-precipitate with DNA
Chaotropic salt/silica purification (Qiagen PCR columns)- isolated DNA was colorless
PCR Tox.-little impact upon ^{32}P PCR product yield
Lig. Tox.- little to no effect upon ligation of lambda *PstI* fragments
Trans. Tox.- no effect upon ligation/transformation of *Eco*RI-pUC19
PCR Sens.- amplification similar to no dye as a function of template concentration.
shade- marketing

Figure 7. Relative toxicity of various dye candidates. Yields are derived from TCA insoluble counts of ^{32}P containing PCR reactions.



T1, T2 and T3= Taq controls (no dye) as in block and precipitation, numerals are dye number.
per cycle yield calculated assuming $y_{20}=y_1^{20}$ where y_{20} is the 20 cycle yield (measured) and y_1 is the per cycle yield.

Dye	y_{20} (%)	y_1(%)
23	9.04	85.3
24	84.1	99.1
25	88.9	99.4
26	74.6	98.5
31	85.6	99.3
39	82.4	99.0

Figure 8. Relative toxicity of various dye candidates on ligation. DNA is PstI digested lambda at 0.5 micrograms per microliter in 1X ligase buffer and 1mM ATP. Reactions were at 16°C for 1 hour. t_0 is without ligase, CTL is +ligase and - dye, 24,25,26,31,39 are dye candidates at concentrations suitable for gel loading and tracking +ligase.

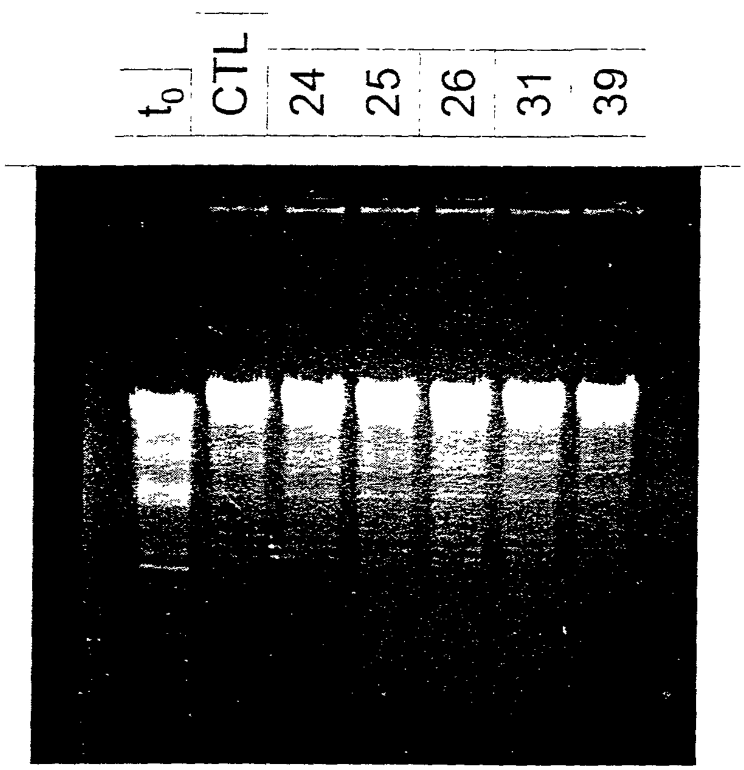


Figure 9. Relative toxicity of various dye candidates on ligation followed by transformation. DNA is HindIII cut pUC19 that was ligated in presence and absence of various dyes. Ligations were as described in Figure 8. Transformation conditions were as described for DH5α competent cells (Life Technologies. -lig is without ligase, +lig is +ligase and - dye, 24,25,26,31,39 are dye candidates at concentrations suitable for gel loading and tracking +ligase.

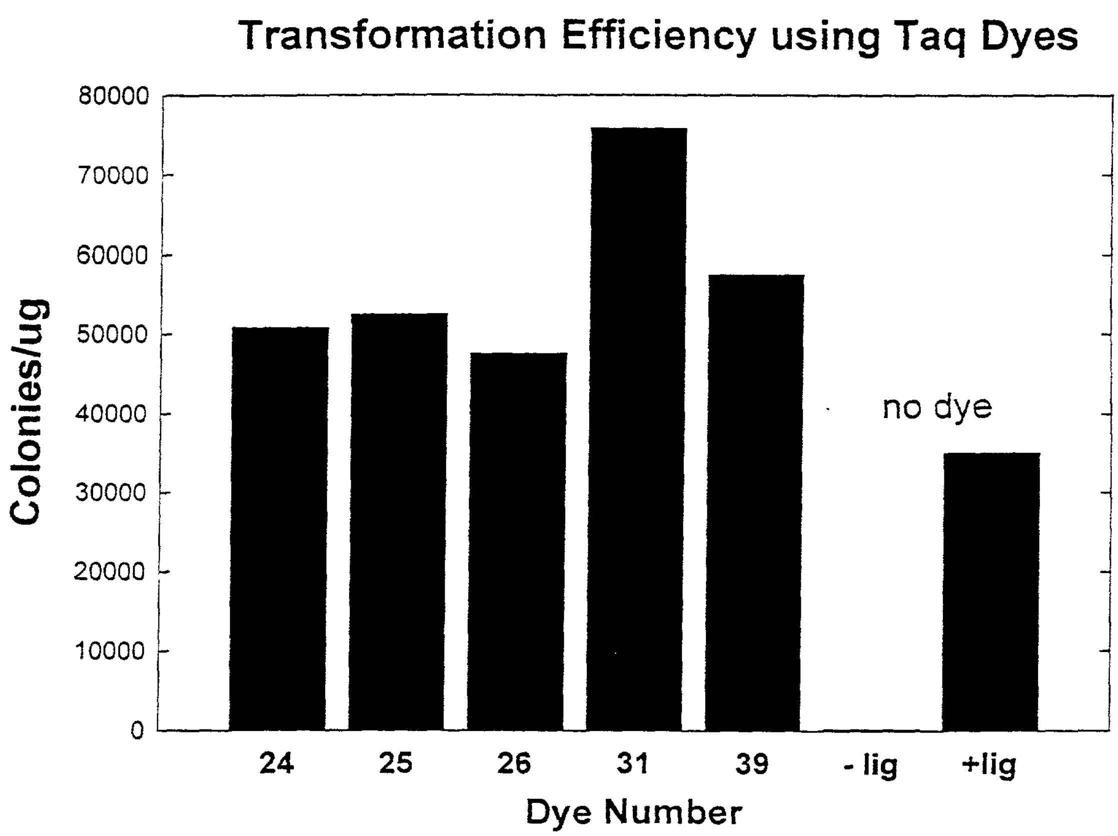


Figure 10. Relative PCR yields using red (acid violet 5) and white Taq DNA polymerase. Target=lambda 500mer (Perkin-Elmer control), 25 cycles (94,55,72°C at 1 min each), [Taq]=0.05 units per microliter, replicate=4. Yield from TCA precipitable counts (^{32}P).

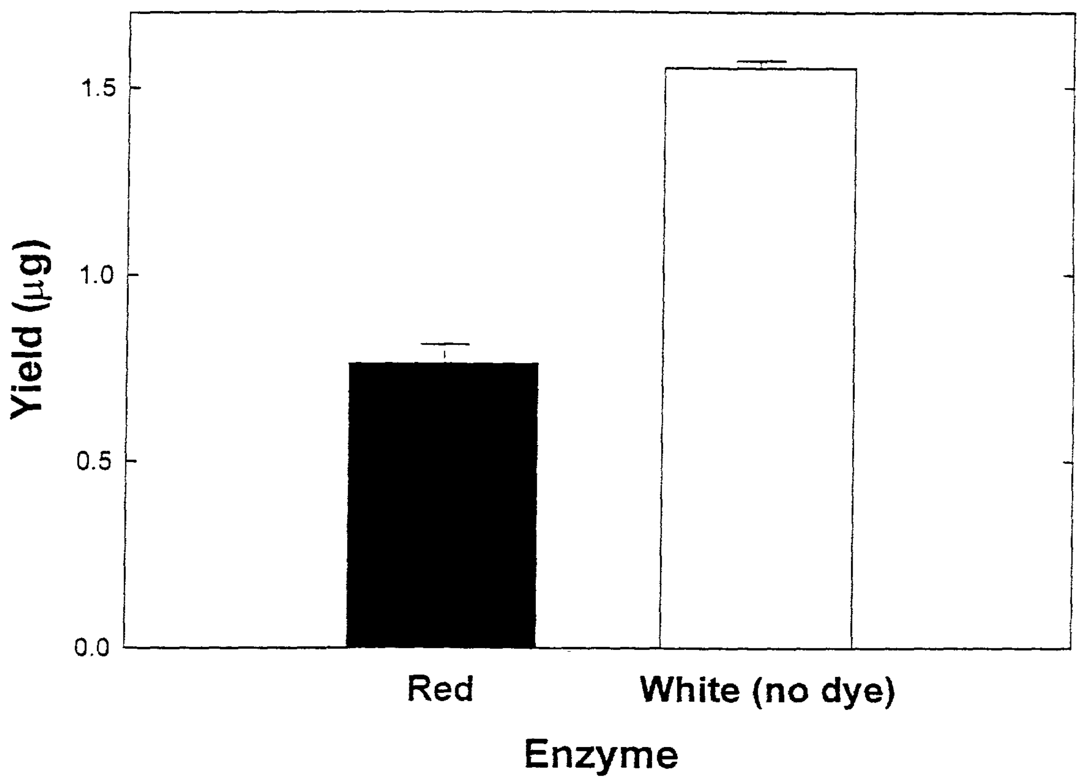


Figure 11. PCR yield of crude vs. pure (desalted, C18 resin, sep-pak, Millipore) acid violet 5 containing reactions. Target and PCR conditions were as described in Figure 10.

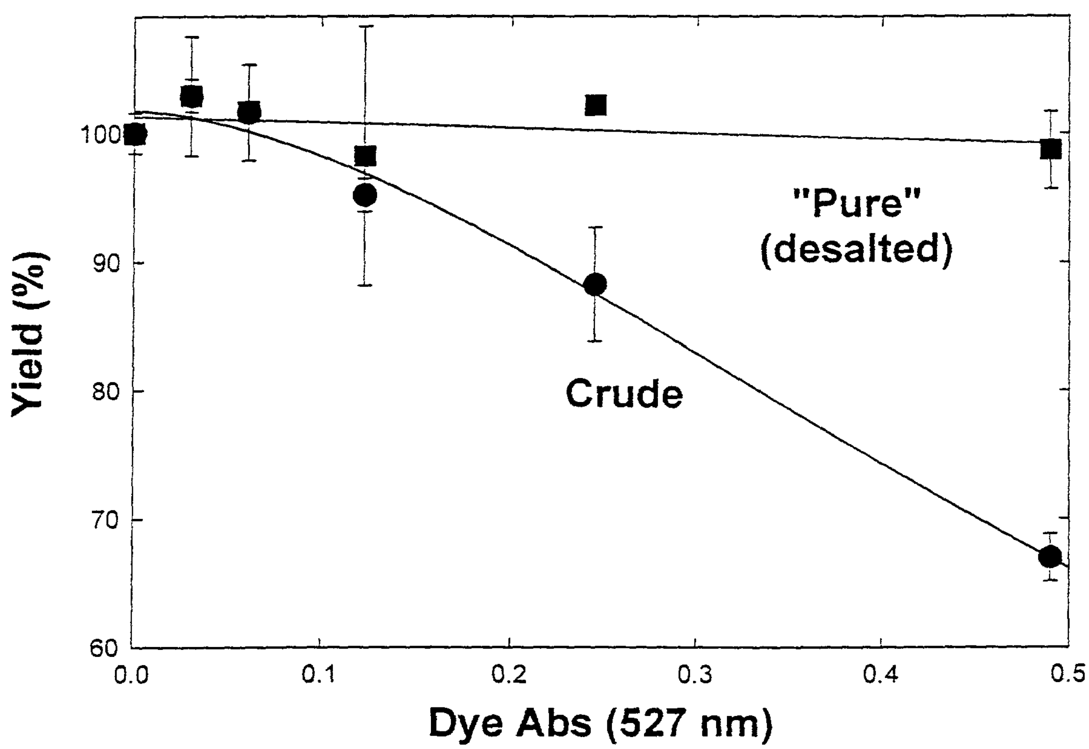


Figure 12a. PCR yields of Mg, Na and NH_4 purified acid red 1 and acid violet 5 dyes. Na purified as in Figure 11. NH_4 prepared by a. HCl pptn, b. NH_4OH solvation c.. Evaporation of excess NH_4OH. Mg dyes prepared by precipitation using $MgCl_2$ (Mg salts much less soluble than Na salts). Target and PCR conditions were as described in Figure 10.

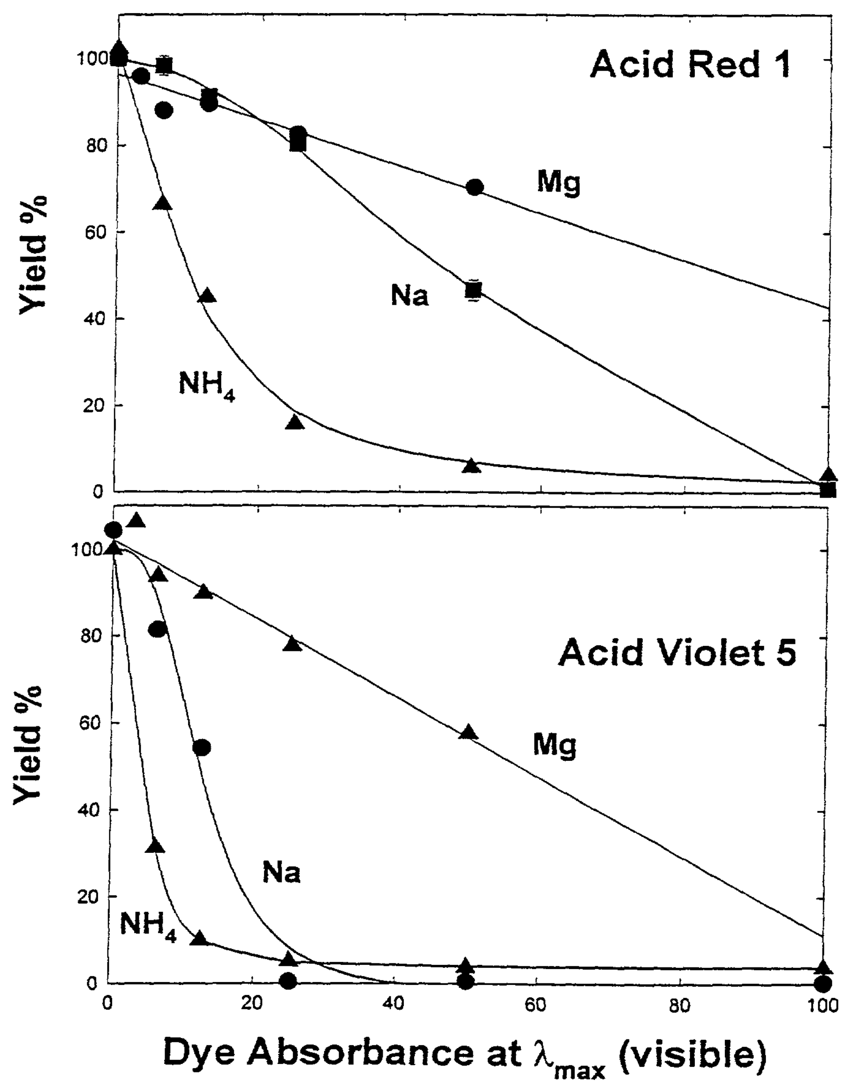


Figure 12b. PCR yields of Mg, Ca and Zn purified acid red 1. Divalent dyes were prepared as for Mg in Figure 12a. Target and PCR conditions were as described in Figure 10.

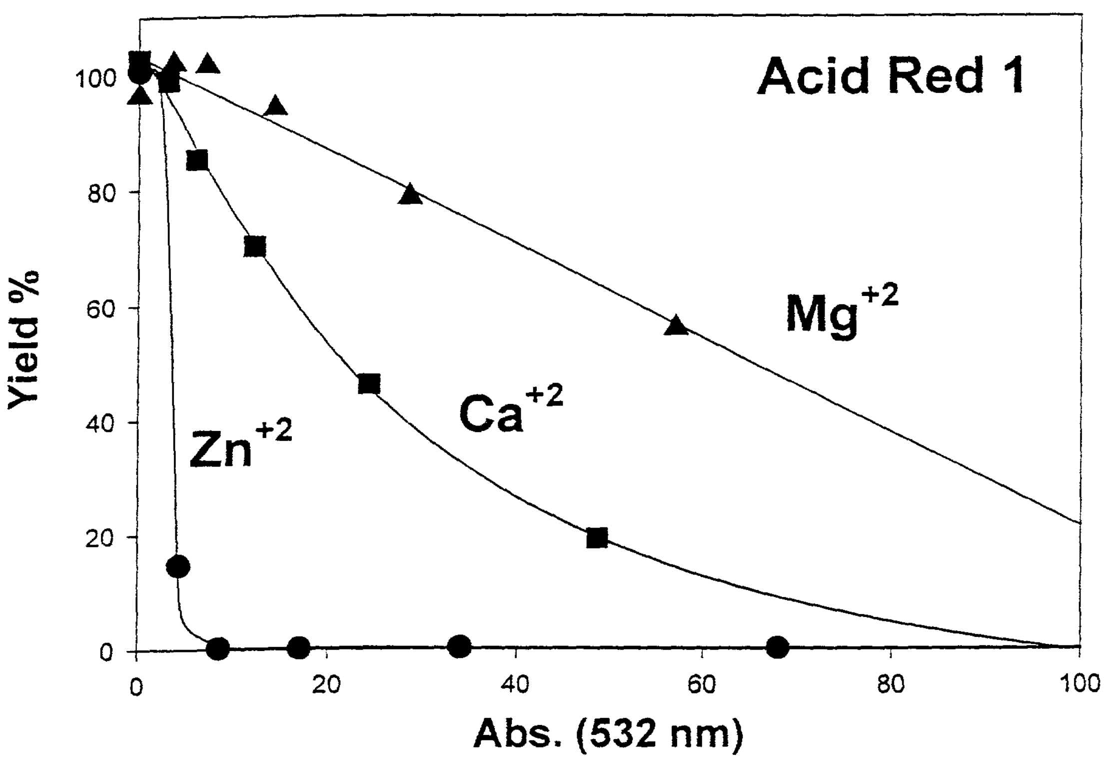


Figure 13. Apparent $[Mg^{+2}]$ of red Taq (80% acid red 1, 20% acid violet 5). $[Mg^{+2}]_{app}$ =0.37+/-0.04mM. Target and PCR conditions were as described in Figure 10. 500, 1500 and 3000mers were different lambda DNA target sizes.

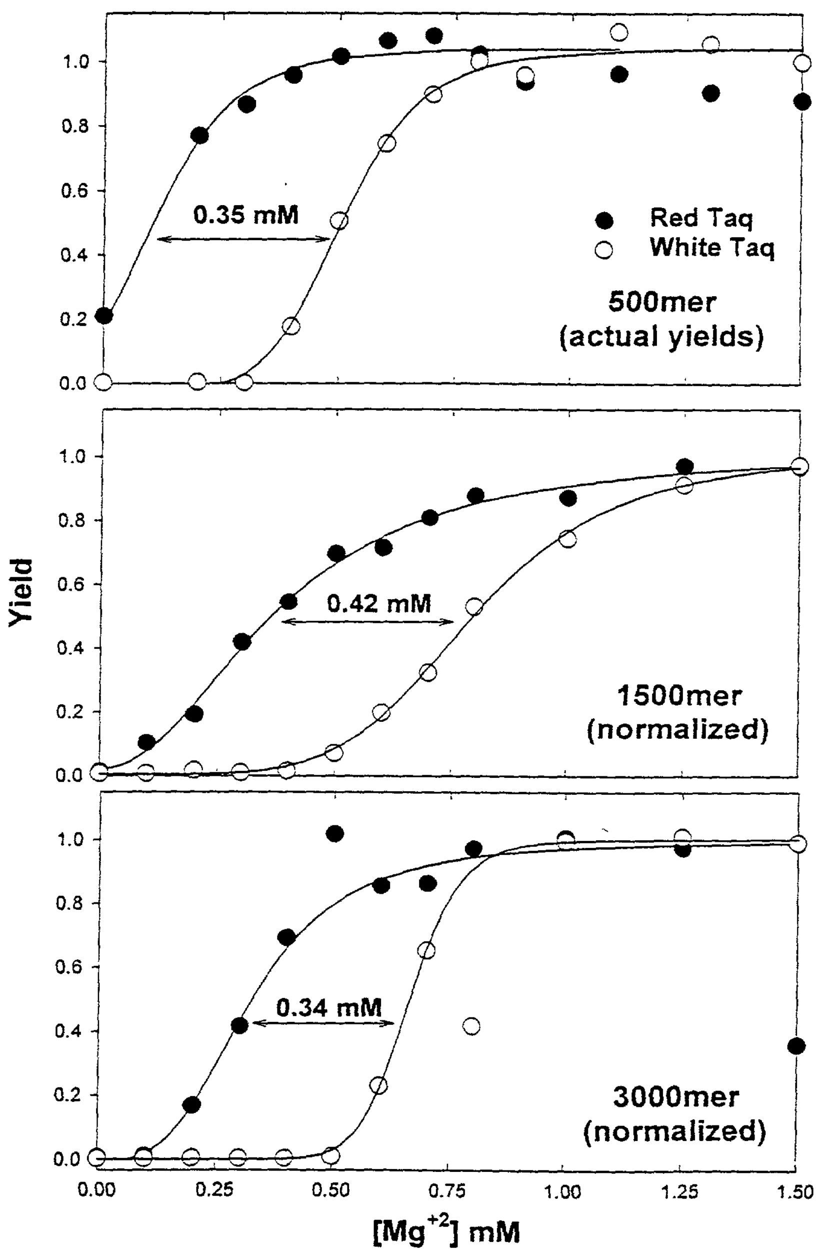


Figure 14. 1% agarose gel contrasting Taq DNA polymerase PCR products formulated without (lanes 2-6) and with (lanes 7-11) optimized analysis reagents. Lane 1,12 are Lambda *Hin*dIII markers. Lanes 2-6 and 7-11 sequentially correspond to amplification targets of length 1,2,3,7 and 10 kb (template=lambda). Absence of product in lane 4 is an artifact.

1 2 3 4 5 6 7 8 9 10 11 12

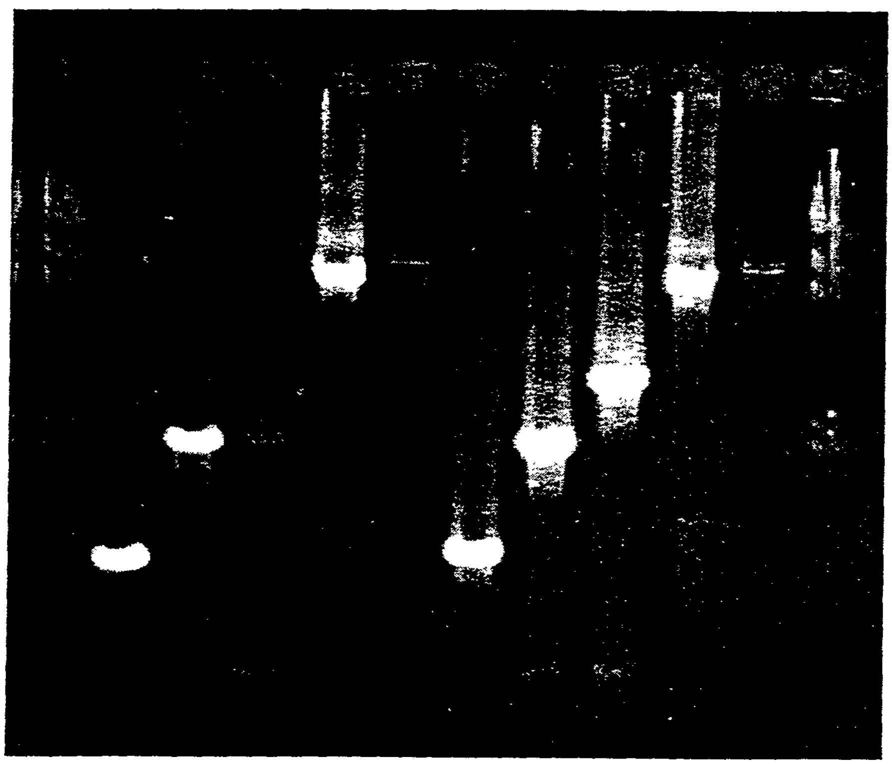


Figure 15. PCR product yields using red and white Taqs with their respective buffers as a function of product length. At each length white Taq was considered 100%, red Taq was relative to white.

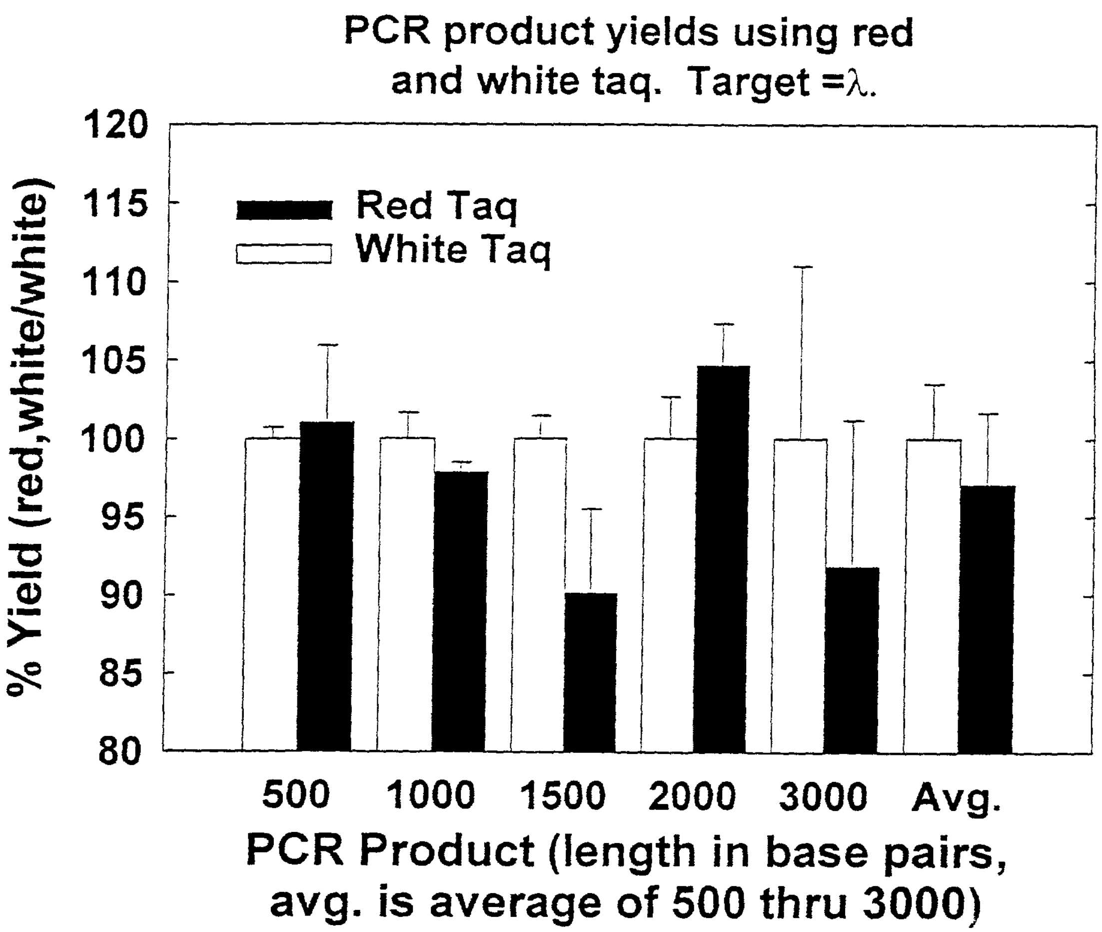


Figure 16. PCR product yields using Taq and Taq + Rediload (Research Genetics). PCR as in Figure 10.

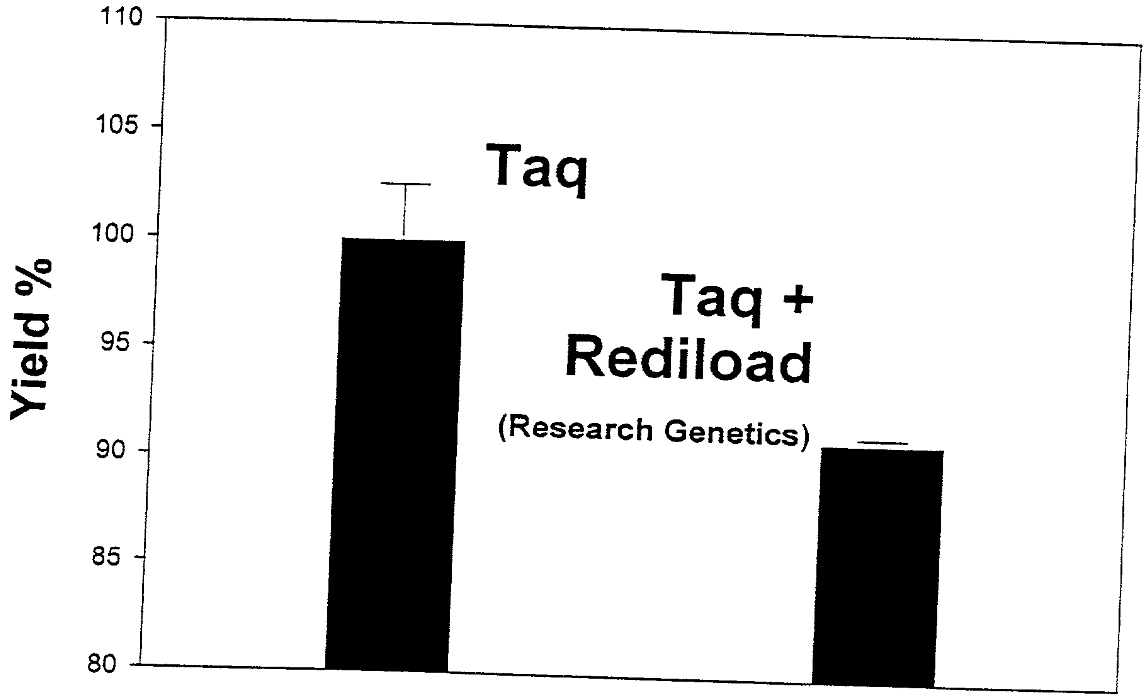
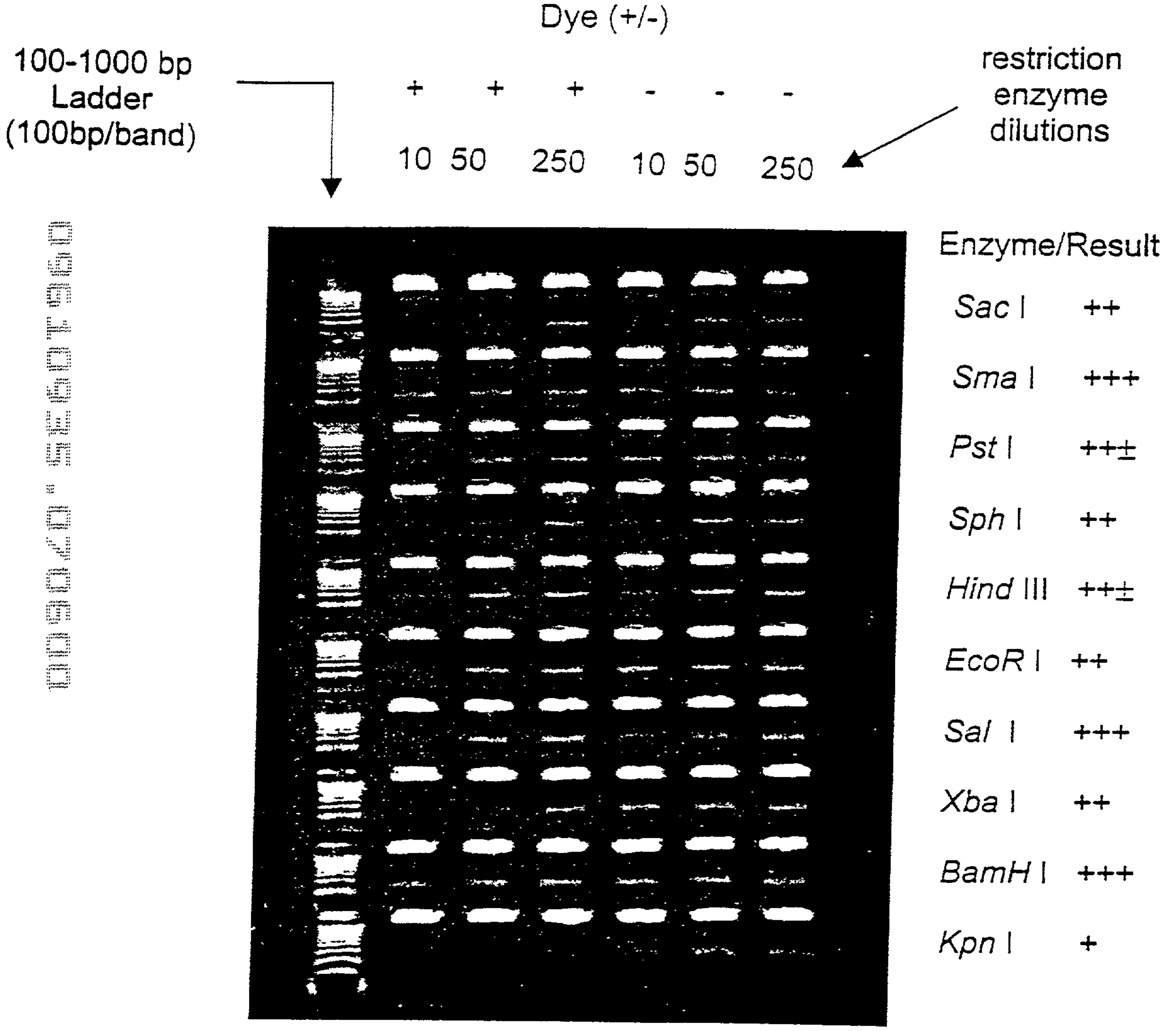


Figure 17. 4% agarose gel electrophoresis of restriction enzyme digestion of NdeI cut pUC19 in presence (sufficient for use as a tracking dye) and absence of crude Amaranth. Column 1 is a 100 to 1000 base pair (100 bp/band) DNA molecular weight ladder. Columns 2-4 are with dye and decreasing amounts of restriction enzyme (I.e. 5X dilution per column), columns 5-7 are as 2-6 except in absence of dye. Rows are labeled with the appropriate restriction enzyme.



Attorney's Docket No. SGM 6934.1

DECLARATION AND POWER OF ATTORNEY

REGULAR OR DESIGN APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TRACER REAGENTS THAT ENHANCE REACTION-PRODUCT ANALYSIS

the specification of which:

(check one)

[X] is attached hereto
[ ] was filed on ____________ as Application Serial No. ___________, and was amended on ____________________.
[ ] was described and claimed in PCT International Application No. ______________, filed on ______________ and as amended under PCT Article 19 on _______________, if any.

ACKNOWLEDGEMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a) - (d) or §365(b) of any foreign application for patent or inventor's certificate, or §365(a) of any PCT application which designates at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Priority Claimed

(Number)	(Country)	(Day/Month/Year Filed)
(Number)	(Country)	(Day/Month/Year Filed)
(Number)	(Country)	(Day/Month/Year Filed)

Priority Not Claimed

ANY FOREIGN APPLICATION(S), ON THE SAME SUBJECT MATTER WHICH HAS A FILING DATE EARLIER THAN THE EARLIEST APPLICATION FROM WHICH PRIORITY IS CLAIMED

(Number)	(Country)	(Day/Month/Year Filed)

CLAIM FOR BENEFIT OF PROVISIONAL APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

60/143,009	07/09/99
(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

CLAIM FOR BENEFIT OF EARLIER U.S. APPLICATION(S) UNDER 35 U.S.C. 120

(complete this part only if this is a divisional, continuation or CIP application)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Serial No.)	(Filing Date)	(Status)
(Serial No.)	(Filing Date)	(Status)

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

- -

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